

Original article

IgE antibodies of fish allergic patients cross-react with frog parvalbumin

Background: The major allergens in fish are parvalbumins. Important immunoglobulin (Ig)E cross-recognition of parvalbumins from different fish species has been shown. Recently frog parvalbumin α has been found to be responsible for a case of IgE-mediated anaphylaxis triggered by the ingestion of frog meat. The aim of this study was to investigate whether IgE antibodies of fish allergic persons cross-react with frog parvalbumin and to appreciate its clinical relevance.

Methods: The sera of 15 fish allergic patients and one fish and frog allergic patient were tested by IgE-immunoblotting against frog muscle extract. Sera were tested against recombinant parvalbumin α and β from *Rana esculenta*. Skin prick tests were performed in selected patients with recombinant frog parvalbumin. Ca^{2+} depletion experiments and inhibition studies with purified cod and frog recombinant parvalbumin were done to characterize the cross-reactive pattern.

Results: Fourteen of the sera tested had IgE antibodies recognizing low molecular weight components in frog muscle extract. Calcium depletion experiments or inhibition of patient sera with purified cod parvalbumin led to a significant or complete decrease in IgE binding. When tested against recombinant parvalbumins, three of 13 sera reacted with α parvalbumin and 11 of 12 reacted with β parvalbumin from *R. esculenta*. Skin prick tests performed with recombinant frog parvalbumin were positive in fish allergic patients. Inhibition studies showed that a fish and frog allergic patient was primarily sensitized to fish parvalbumin.

Conclusion: Cod parvalbumin, a major cross-reactive allergen among different fish species, shares IgE binding epitopes with frog parvalbumin. This *in vitro* cross-reactivity seems to be also clinically relevant. Parvalbumins probably represent a new family of cross-reactive allergens.

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The prevalence of food allergies in European countries varies considerably and has recently been estimated to lie between 3% (1) and 16% (2). In these surveys were included primary food allergies and food allergies because of cross-sensitization with tree or weed pollen allergens. Dairy products, peanuts, nuts, eggs, cereals, crustaceans and fish are the most important sources of primary food allergens. In codfish the major allergen is Gad c1 (3). The homologous allergens have been isolated and the corresponding cDNAs have been cloned from atlantic salmon (*Salmo salar*) (4), carp (*Cyprinus carpio*) (5, 6) and atlantic cod (*Gadus morhua*) (7, 8). They belong to the parvalbumins, a family of calcium binding proteins which play an important role in muscle physiology. Parvalbumins contain 3 EF-hand motifs (two perpendicularly placed α helices E and F which form together with an interhelical loop a single Ca^{2+} binding site) of which only

the second and third domains are able to bind calcium (9). They are divergent proteins in terms of primary structure. More than one isotype can be found in the same muscle. Based on amino acid sequence data, two distinct phylogenetic lineages of parvalbumins, named α and β , have been recognized in higher and lower vertebrates (10). They are low molecular weight (≈ 12 kD), acidic, water soluble proteins with a high resistance to thermal and enzymatic degradation (11, 12). In carp it was shown that the protein is abundantly distributed in muscle fiber cells (13). By inhibition experiments it was demonstrated that the protein could inhibit 78–89% of the total IgE binding activity to extracts of cod fish, tuna and salmon (13). Amino acid sequences of parvalbumin from several fish species have been determined by protein sequencing. These show significant sequence homology.

Recently we have isolated the cDNAs coding for parvalbumin α and β from *Rana esculenta* (14). A patient reacting with an anaphylactic shock after ingestion of fried frog legs had IgE antibodies to parvalbumin α from the frog species that provoked the allergic reaction. Frog parvalbumin α and β have an amino acid sequence identity of 45–70% to parvalbumins from different fish species. In the present study we tested whether a series of fish allergic patients and a previously published (15) highly atopic person with combined allergy to fish and frog had cross-reactive IgE antibodies to fish and frog parvalbumins. IgE Immunoblotting using cod and *R. esculenta* protein extracts, purified cod parvalbumin and recombinant frog parvalbumin, combined to calcium depletion and immunoblot and enzyme-linked immunosorbent assay (ELISA) allergen inhibition experiments were performed.

Methods

Human sera

Sera from 16 patients with specific IgE antibodies (Pharmacia CAP-System; Pharmacia, Uppsala, Sweden) to cod were selected for this study (Table 1). One of the patients (No. 15, Table 1) known to be allergic to fish and frog has been the subject of a previous publication (15).

Material

Rana esculenta was obtained from a breeding center. Frog muscle was immediately frozen on dry ice and stored at -80°C . Cod was purchased in a local supermarket. Recombinant parvalbumin α - and β were prepared and purified as described (14).

Table 1. Serologic characterization of fish allergic patients Cod Salmon Tuna

Patient	Total IgE concentration kU/l	Specific IgE binding to fish by CAP kU/l		
		Cod	Salmon	Tuna
1	664	40.8	58.7	21.6
2	2603	>100	>100	4.9
3	>1000	88.1	42.3	4.5
4	509	33.7	14.3	15.7
5	366	7.5	8	4.2
6	14585	17.2	5.3	6.5
7	451	3.7	2.4	<0.35
8	1484	11	8.9	0.8
9	1521	92.5	nd	nd
10	323	4.9	12.4	4.8
11	790	31.6	13.7	0.5
12	1927	4.5	nd	nd
13	240	2.9	4.9	nd
14	633	75	88	42
15	2950	>100	>100	89
16	156	3.9	4.3	1.1

nd, Not done.

Preparation of protein extracts

Frog skeletal muscle and fish muscle were disrupted in single detergent lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100, 100 $\mu\text{g/ml}$ Phenylmethylsulfonyl fluoride (PMSF) (Sigma, St Louis, MO) by a rotor-stator homogenizer (Kinematica, Luzern, Switzerland). The extract was centrifuged for 10 min at 5300 g to remove insoluble particles and the protein concentration of the supernatant was determined by Bio-Rad protein assay (Bio-Rad, Nazareth, Belgium). Cod parvalbumin was purified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as follows. Cod extract was centrifuged for 1 h at 50 000 g at 4°C and concentrated with an Ultrafree concentrator (Millipore, Bedford, MA). Parvalbumin was purified from 40 mg of protein on a MiniPrep Cell (Bio-Rad). Eluted proteins were analyzed by SDS-PAGE followed by silver staining (Amersham Pharmacia, Uppsala, Sweden). Fractions were pooled and concentrated as above by centrifugation.

SDS-PAGE and detection of specific IgE antibodies by immunoblot

Proteins were separated by 18% SDS-PAGE gels under denaturing conditions and visualized by Coomassie brilliant blue colloidal staining. For immunoblotting proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). To reduce nonspecific binding, blotted membranes were incubated in blocking buffer (TBS/Tween-20 0.05%, 2% cold water fish gelatine (Sigma)). The PVDF membrane was cut into strips which were incubated overnight with patients' sera diluted one-tenth in blocking buffer. Control strips were incubated with the serum of a grass-pollen allergic patient or buffer alone. Strips were washed four times with TBS/Tween-20 0.05%. Bound IgE were detected with anti-human IgE antibody labeled with alkaline phosphatase (Sigma) and the blots were developed by addition of nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Promega, Madison, WI).

Detection of parvalbumin by mouse monoclonal antibody

Allergens were separated by 18% SDS-PAGE gels and blotted onto PVDF membranes as described above. After blocking nonspecific binding sites with 2% cold water fish gelatine, the membranes were incubated with a mouse monoclonal antibody directed against frog skeletal muscle parvalbumin (clone Parv-19, Sigma) diluted in blocking buffer. The bound mouse monoclonal antibody was detected with anti-mouse IgG linked to horseradish peroxidase (Amersham Pharmacia). Blots were developed by addition of diaminobenzidine (Sigma).

Ca²⁺ depletion experiments

Frog muscle extract was separated by 18% SDS-PAGE under denaturing conditions with β mercaptoethanol and transferred onto a PVDF membrane. Sera were diluted one-fifth in blocking buffer in the presence of 0.5 mM CaCl_2 or 5 mM EGTA, pH 7.5 and incubated with membrane strips as described above.

ELISA inhibition experiments

The ELISA inhibition experiments were performed with purified recombinant *R. esculenta* parvalbumin α and β and purified cod parvalbumin. Parvalbumins were coated at a concentration of 5 $\mu\text{g/ml}$ in phosphate-buffered saline (PBS) to microtiter plates

overnight at 4°C. Residual binding sites were saturated by incubation with blocking buffer (2% cold water fish gelatine in PBS/0.1% Tween-20) for 1 h. Patient sera diluted in blocking buffer (one-fifth for detection of parvalbumin α , one-tenth for parvalbumin β) were preincubated for 2 h with 40 μ g cod parvalbumin or BSA before addition to the microtiter plate for 2 h. Binding of IgE was measured by incubation for 1 h with biotin labeled anti-human IgE antibodies (1/2000; Southern Biotechnology, Birmingham, AL), followed by an incubation for 30 min with alkaline phosphatase labeled streptavidine (1/1000; Pharmingen, Erembodegem, Belgium). p-NPP was added as a substrate and plates were left in the dark for approximately 30 min before optical density was read at 405 nm. All determinations were done in duplicate. The percentage of inhibition of IgE binding was calculated from the mean OD values measured with or without addition of inhibitor according to the following formula: % inhibition = $100 - \text{OD}_{\text{inhibitor}} \times 100 / \text{OD}_{\text{control}}$.

IgE immunoblot inhibition

Serum from patient no. 15 with specific IgE to fish was diluted one-tenth in blocking buffer and incubated either with 20 μ g of purified parvalbumin or, as a control, with 20 μ g of BSA for 2 h at room temperature. Subsequently serum was exposed overnight to PVDF membrane strips with blotted frog or cod parvalbumin and processed as described above.

Skin prick testing

After informed consent was obtained from three fish allergic patients and two non-allergic individuals, skin prick tests were performed on the forearms of the patients. Individuals were pricked with increasing concentrations of recombinant parvalbumins (0.1, 1, 10 μ g/ml for parvalbumin α ; 0.07, 0.7, 7 μ g/ml for parvalbumin β) diluted in 0.03% human albumin, 0.9% NaCl, 0.4% Phenol (ALK ABELLO, Hørsholm, DK). Histamine was used as a positive control and the diluent as negative control. Skin reactions were recorded 20 min after testing. Positive results were graded as follows: (+) when the diameter of the wheal was 3–5 mm, (++) for 5–10 mm, and (+++) for 10–15 mm. Recombinant *R. esculenta* parvalbumin α and β were produced in *Escherichia coli* M15 and purified as described (14). Proteins were dialyzed against PBS and sterile filtered.

Results

IgE recognition pattern of fish and frog muscle extracts by sera of fish allergic patients

Sixteen patients with specific IgE to cod (see Table 1) were selected to investigate the IgE binding to cod and frog muscle extract. The sera of the patients with fish allergy, and as controls, serum from a grass-pollen allergic patient and buffer without serum, were tested for IgE reactivity with blotted cod or frog muscle extract (Fig. 1). Fifteen sera recognized cod proteins of high and low molecular weight, the latter including probably the 12 kD protein parvalbumin (Fig. 1A). Fourteen sera contained IgE antibodies directed against low molecular weight components of frog muscle extract, presumably including parvalbumin (Fig. 1B). A mouse monoclonal antibody directed against frog parvalbumin (Parv-19)

recognizes frog parvalbumin in the frog muscle extract at 12 kD.

IgE recognition of frog parvalbumin is Ca^{2+} dependent

Parvalbumins are Ca^{2+} -binding proteins. It has been shown that IgE binding to parvalbumin can be modulated by the presence or absence of Ca^{2+} (13). To investigate whether cross-reactive IgE binding epitopes of parvalbumin require protein-bound calcium, PVDF membrane strips with blotted frog muscle extract were exposed to CaCl_2 or EGTA during IgE immunoblotting (Fig. 2). Sera from seven patients with specific IgE antibodies to fish (and for whom enough serum was available) were incubated with blotted frog muscle extract in the presence or absence of calcium. A significantly reduced or even totally abolished IgE binding was observed on Ca^{2+} depletion of the sera. The recognition of frog parvalbumin by mouse monoclonal anti-frog parvalbumin antibody Parv-19 is also Ca^{2+} dependent.

IgE antibodies of fish allergic patients recognize recombinant frog parvalbumin

cDNAs coding for parvalbumin α and β had been isolated previously from *R. esculenta* (14). Both cDNAs were expressed in *E. coli*. The sera of patients allergic to fish were tested for IgE recognition of recombinant parvalbumin α and β from *R. esculenta*. Sera were incubated with membrane strips carrying blotted recombinant parvalbumins (Fig. 3). Three of 13 patients had IgE antibodies reacting with parvalbumin α ; 11 of 12 patients reacted with parvalbumin β . Mouse monoclonal antibody Parv-19 directed against frog parvalbumin recognizes recombinant parvalbumin α and β of *R. esculenta*. In Fig. 3B a band of about 28 kD appearing in several lanes (2, 5, 7, 9, Parv-19) is probably because of uncomplete sample denaturation as this band is missing on other strips done in different experiences (lanes 13–16).

ELISA inhibition with purified cod parvalbumin

The ELISA inhibition experiments were performed to further evaluate the cross-reactivity between cod and frog parvalbumin. Recombinant parvalbumin α or β coated to the wells of a microtiter plate were incubated with the serum of fish allergic patients in the presence or absence of purified cod parvalbumin (Fig. 4). Sera 2 and 15 did not show IgE reactivity to parvalbumin α . The IgE reactivity of serum 14, which was weakly positive for parvalbumin α was completely inhibited by purified cod parvalbumin. Recombinant parvalbumin β was recognized by the three patient sera analyzed and IgE binding was abolished by inhibition with purified cod parvalbumin (inhibition 96–99.7%).

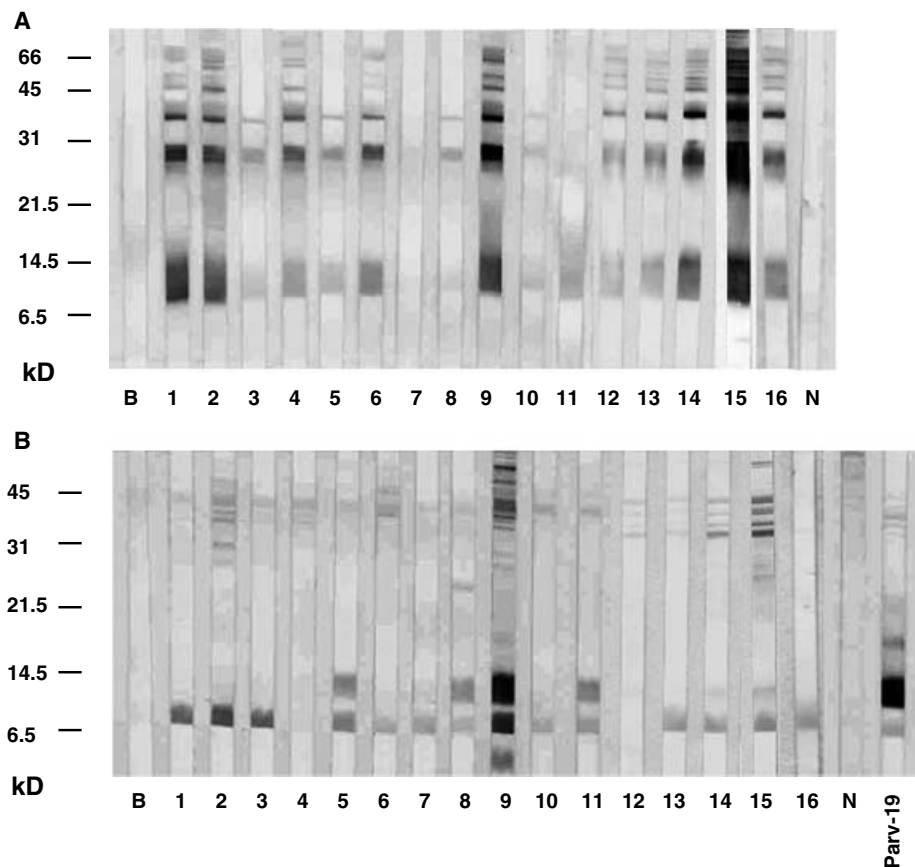


Figure 1. Immunoglobulin (Ig)E binding to cod and frog muscle extract. Cod protein extract (A) and frog muscle extract (B) were separated by SDS-PAGE and immunoblotted onto polyvinylidene difluoride membranes. Sera from 15 fish allergic patients (lanes 1–14 and 16) and one fish and frog allergic patient (lane 15) were tested for IgE reactivity to cod and frog proteins. Serum from a grass-pollen allergic patient (lane N) and buffer without serum (lane B) were used as controls. Parv-19 is a mouse monoclonal anti-frog parvalbumin antibody.

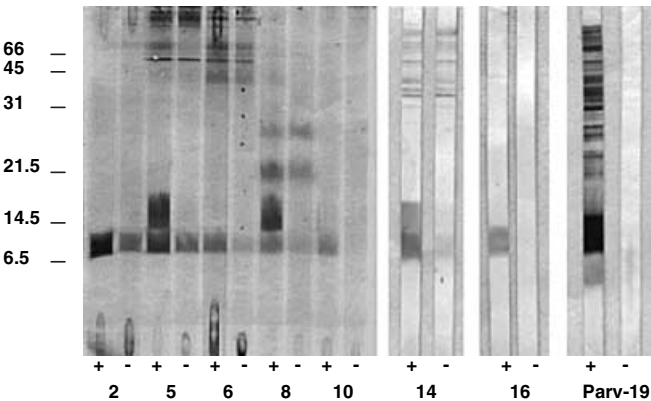


Figure 2. Immunoglobulin E binding to frog muscle extract is Ca²⁺ dependent. Sera from seven fish allergic patients (lanes 2, 5, 6, 8, 10, 14, 16) were exposed to blotted frog muscle extract in the presence of CaCl₂ (+ lanes) or EGTA (– lanes). Parv-19 is a mouse monoclonal antibody raised against frog parvalbumin.

Cross-reactivity between fish and frog parvalbumin in a case of IgE-mediated anaphylaxis after ingestion of frog meat

The serum of a fish allergic child who had suffered from an IgE-mediated anaphylaxis after ingestion of boiled frog meat (15) was analyzed for cross-reactivity between fish and frog parvalbumin (serum 15, Table 1). The serum contained specific IgE antibodies to cod and frog proteins of low molecular weight (Fig. 1). The IgE antibodies of the patient recognized recombinant parvalbumin β (Fig. 3) from *R. esculenta*.

To elucidate the nature of the cross-reactivity, cross-inhibition experiments were performed with purified cod parvalbumin and purified recombinant frog parvalbumin β . In Fig. 5A the serum is incubated with membrane strips with blotted recombinant frog parvalbumin β and inhibited with either BSA or cod parvalbumin. Figure 5B shows the incubation of the serum with membrane strips with blotted cod parvalbumin, inhibited with either BSA or recombinant frog parvalbumin β . Cod parvalbumin completely abolished the IgE antibody recognition of

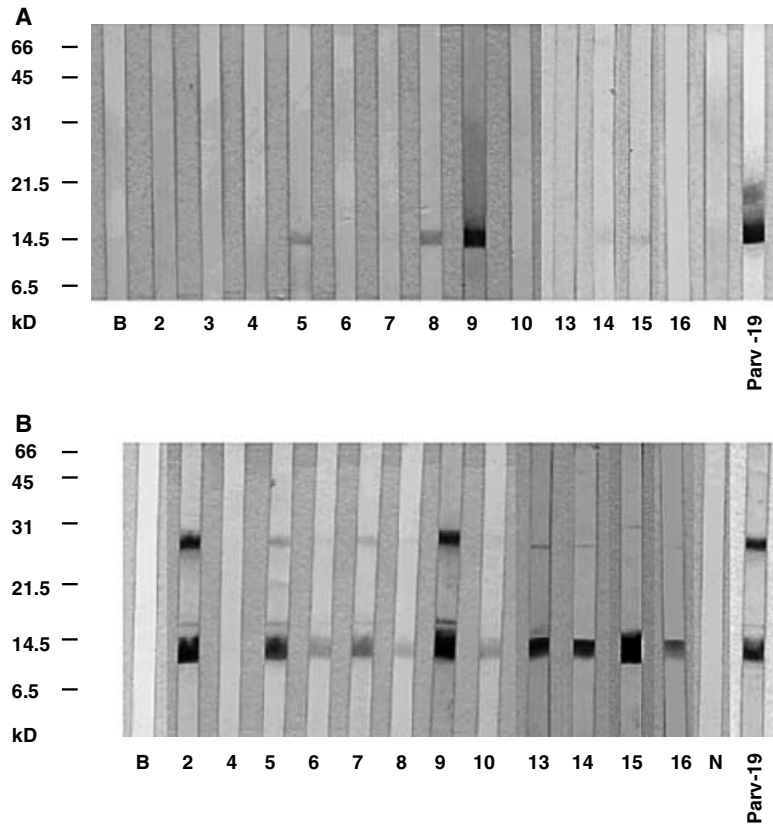


Figure 3. Immunoglobulin E binding to recombinant frog parvalbumin. Polyvinylidene difluoride membrane strips with blotted recombinant parvalbumin α (A) or β (B) from *Rana esculenta* were incubated with different sera: lanes 2–16, fish allergic patients. Serum from a grass-pollen allergic patient (lane N), blocking buffer without serum (lane B) and mouse monoclonal antiparvalbumin antibody Parv-19 were used as controls.

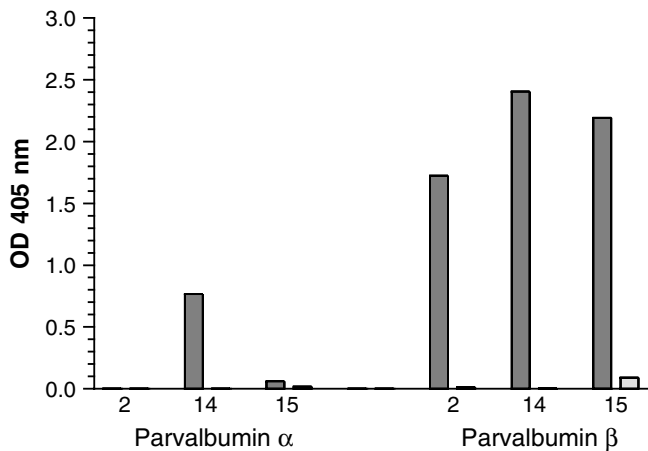


Figure 4. Inhibition of immunoglobulin (Ig)E binding to recombinant frog parvalbumin. Sera from two fish allergic patients (patients 2, 14) and one fish and frog allergic patient (15) were tested by enzyme-linked immunosorbent assay for IgE reactivity to recombinant *Rana esculenta* parvalbumin α and β after preincubation with bovine serum albumin (control; dark grey bars) or purified cod parvalbumin (light grey bars). The y-axis displays the extinctions measured as optical density (OD) at 405 nm.

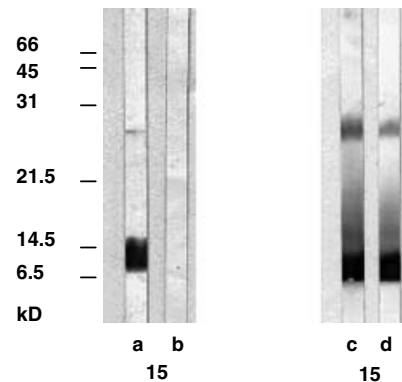


Figure 5. Inhibition of immunoglobulin (Ig)E binding to frog and cod proteins assayed with the serum of a fish allergic patient who made an anaphylactic reaction upon ingestion of frog meat. Polyvinylidene difluoride membrane strips with blotted recombinant *Rana esculenta* parvalbumin β (lanes a, b) or purified cod parvalbumin (lanes c, d) were incubated with serum no. 15 (see Table 1 for details). IgE binding was inhibited with purified cod parvalbumin (lane b) or recombinant frog parvalbumin β (lane d). In lanes a and c inhibitions were performed with bovine serum albumin as control.

	1				50		
p02617;prvb_ranes	MSITDI	VSEK	DIDAALESVK	AAGSFNYKIF	FQKVGLAGKS	AADAKKVFEI	
p02618;prvb_cypca	~AFAGV	LND	DI	AAALEACK	AADSFNHKAF	FAKVGLTSKS	ADDVKKAF
p09227;prva_cypca	~AYGGI	LND	DITAALEACX	AXDSFNAKSF	FAKVGLSAKT	PDDIKKAF	
p02620;prvb_merme	~AFAGIL	ADA	DITAAALACK	AEGSEFKHGEF	FTKIGLKGKS	AADIKKVFGI	
p02621;prvb_mermr	~AFAGIL	ADA	DCAAAVKACE	AADSFYSYKAF	FAKCGLSGKS	ADDIKKAFVF	
p56503;prvb_merbi	~AFSGI	LADA	DVAAALKACE	AADSFNYKAF	FAKVGLTAKS	ADDIKKAFFV	
q91482;prv1_salsa	MACAHLCKEA	DIKTALEACK	AADTF	SFKTF	FHTIGFASKS	ADDVKKAFKV	
q91483;prv2_salsa	~SFAGL	LND	DVAAALAACT	AADSFNHKAF	FAKVGLASKS	SDDVKKAFYV	
p02622;prvb_gadca	~AFKGI	LSD	DIKAAEAACF	KEGSFDEDDGF	YAKVGLDAFS	ADELKKLFKI	
p02619;prvb_esolu	~SFAGL	KDA	DVAAALAAACS	AADSFKHKEF	FAKVGLASKS	LDDVKKAFYV	
	51					100	
p02617;prvb_ranes	LDRDKSGFIE	QDELGLFLQN	FRASARVLS	D	AETSAFLKAG	DSDGDGKIGV	
p02618;prvb_cypca	IDQDKSGFIE	EDELKLFLQN	FKADARALTD	GETKTFLKAG	DSDGDGKIGV		
p09227;prva_cypca	IDQDKSGFIE	EDELKLFLQN	FSAGARALTD	AETKAFLKAG	DSDGDGKIGV		
p02620;prvb_merme	IDQDKSDFVE	EDELKLFLQN	FSAGARALTD	AETATFLKAG	DSDGDGKIGV		
p02621;prvb_mermr	IDQDKSGFIE	EDELKLFLQV	FKAGARALTD	AETKAFLKAG	DSDGDGAIGV		
p56503;prvb_merbi	IDQDKSGFIE	EDELKLFLQV	FSAGARALTD	AETKAFLKAG	DSDGDGAIGV		
q91482;prv1_salsa	IDQDASGFIE	VEELKLFLQN	FCKPARELTD	AETKAFLKAG	DADGDMIGI		
q91483;prv2_salsa	IDQDKSGFIE	EDELKLFLQN	FSASARALTD	AETKAFLADG	DKDGDGMIGV		
p02622;prvb_gadca	ADEDKEGFIE	EDELKLFLIA	FAADLRALTD	AETKAFLKAG	DSDGDGKIGV		
p02619;prvb_esolu	IDQDKSGFIE	EDELKLFLQN	FSPSARALTD	AETKAFLADG	DKDGDGMIGV		
	101		114				
p02617;prvb_ranes	EEFQALVKA*	~~~~					
p02618;prvb_cypca	DEFTALVKA~	~~~~					
p09227;prva_cypca	DEFAALVKA~	~~~~					
p02620;prvb_merme	EEFAAMVKG~	~~~~					
p02621;prvb_mermr	EEWVALVKA~	~~~~					
p56503;prvb_merbi	DEWAALVKA~	~~~~					
q91482;prv1_salsa	DEFAVLVKQ~	~~~~					
q91483;prv2_salsa	DEFAAMIKG~	~~~~					
p02622;prvb_gadca	DEFGALVDKW	GAKG					
p02619;prvb_esolu	DEFAAMIKA~	~~~~					

Figure 6. Alignment of *Rana esculenta* parvalbumin β amino acid sequence with different fish parvalbumin sequences. Amino acid identities to *R. esculenta* parvalbumin β are shown in light grey. The two calcium binding sites are underlined. Respective sequence identities to *R. esculenta* are as follows: carp p02618 70% and p09227 68%; European hake p02620 68%; whiting p02621 66%; silver hake p56503 64%; Atlantic salmon q91482 60% and q91483 63%; Baltic cod p02622 60%; Northern pike p02619 62%.

frog parvalbumin while in the reverse experiment addition of frog parvalbumin has hardly any effect on the IgE recognition of cod parvalbumin.

Skin prick testing with recombinant *R. esculenta* parvalbumin α and β

To further investigate the *in vivo* allergenic reactivity of recombinant parvalbumin α and β , skin prick tests were performed. Three fish allergic patients and two nonallergic individuals were skin prick tested with different dilutions of recombinant parvalbumin. Patient 14 was positive for parvalbumin α at a concentration of 10 $\mu\text{g/ml}$ (+ +) and for parvalbumin β at 0.7 $\mu\text{g/ml}$ (+ +). Patient 15 was doubtfully positive for parvalbumin α at a concentration of 10 $\mu\text{g/ml}$ (+ +) and clearly positive for parvalbumin β at a concentration of 0.07 $\mu\text{g/ml}$ (+), 0.7 $\mu\text{g/ml}$ (+ +) and 7 $\mu\text{g/ml}$ (+ + +). Patient 16 was negative for parvalbumin α and positive for parvalbumin β at a concentration of 0.07 $\mu\text{g/ml}$ (+ +).

and 0.7 $\mu\text{g/ml}$ (+ + +). The two nonallergic individuals did not show any reaction to the recombinant parvalbumins.

Sequence comparison of the deduced frog parvalbumin aa sequences with other parvalbumin sequences

Recombinant parvalbumin β from *R. esculenta* was recognized by most sera of fish allergic patients. The protein sequences of parvalbumin α and β of *R. esculenta* were compared with different fish parvalbumin sequences. Figure 6 shows the alignments of frog parvalbumin β with different fish parvalbumins. The most conserved regions include the Ca^{2+} -binding motifs EF1 and EF2. Sequence identities between frog parvalbumin β and fish parvalbumin are in the range of 60–70%, the identity to cod being 60%.

Sequence identities between frog parvalbumin α and different fish parvalbumins lie in the range of 45% (cod) to 54% (salmon).

Discussion

Parvalbumins have been shown to be major allergens in fish. They are responsible for an *in vitro* IgE cross-reactivity between several fish species (16, 17). This cross-reactivity has been shown to be clinically relevant (18). Parvalbumins are low molecular-weight Ca^{2+} binding acidic proteins found in the cytosol of fast twitch skeletal muscles and in nervous tissue (19). Parvalbumin α and β have been shown to coexist in frog (20). We have recently defined frog parvalbumin α as the molecule implicated in a case of anaphylactic shock provoked by the ingestion of fried frog legs. Allergic reactions upon fish consumption are a common problem in certain countries (21) whereas allergic reaction to frog meat have only occasionally been reported. (14, 15, 22–26). The present paper addresses the question of possible IgE cross-reactions and sensitization from fish to frog parvalbumin in fish allergic patients. The sera of 15 fish allergic patients and one fish and frog allergic patient were first tested for IgE antibodies on cod and frog muscle extract. Fourteen of these sera recognized one or two low molecular weight proteins in the frog muscle extract. These bands had a molecular weight corresponding to parvalbumins. A monoclonal anti-parvalbumin antibody recognized protein bands at the same molecular level. Depletion of calcium significantly reduced the IgE binding to the frog muscle extract. Recombinant *R. esculenta* parvalbumin α and β were expressed in *E. coli* to address the question whether the IgE cross-reactivity was related to parvalbumin α or β . Reactivity to parvalbumin α was rather low with only three positive sera of 13 sera tested whereas 11 of 12 sera with IgE antibodies to frog were positive for parvalbumin β . The ELISA inhibition experiments performed with purified cod parvalbumin showed that cross-reactivity to recombinant frog parvalbumin can be abolished by the presence of an excess of fish parvalbumin. Protein sequence alignments showed a sequence identity of 60% between cod parvalbumin and parvalbumin β of *Rana esculenta*, whereas the sequence identity between cod and parvalbumin α of *Rana esculenta* is only 45%. This might explain the low level of IgE crossreactivity between cod parvalbumin and frog parvalbumin α . As the identities are particularly strong in the conserved regions of the calcium-binding domains, it was tempting to test whether these regions contain cross-reactive epitopes. A confirmation of this might be the high sensitivity of the fish specific IgE antibodies cross-reacting with frog parvalbumin to calcium depletion. Indeed calcium depletion has been shown to modify profoundly the three dimensional structure of parvalbumins and to decrease IgE binding (13).

Some patient sera recognized two low molecular weight bands in frog muscle extract (Fig. 1B) The nature of the second band is not definitely known. It is however sensitive to calcium depletion. It is also noteworthy that the sera which recognize two bands

are also the only sera which recognize frog parvalbumin α in addition to parvalbumin β . Therefore the second band might represent frog parvalbumin α although the calculated molecular weight of both parvalbumins is nearly the same. Additional immunoreactive bands have been observed by others in cod extract (27). They have been attributed to polymer or aggregate formation, complexing of parvalbumin with other proteins or common antigenic epitopes with other proteins.

The clinical relevance of these *in vitro* cross-reactivities is best illustrated by a more thorough analysis of a serum sample of a previously published case (15) of severe anaphylaxis provoked by the consumption of boiled frog meat. This highly atopic child had allergies to milk, eggs, and fish (cod, sole). Upon introduction of boiled frog meat as a protein source, the girl experienced a severe anaphylactic reaction. A RAST assay performed with frog meat was clearly positive. Cross-inhibition experiments now performed with purified cod parvalbumin and recombinant *R. esculenta* parvalbumin β confirmed that the child was primarily allergic to fish. Indeed IgE crossreactivity to parvalbumin β from *R. esculenta*, the frog species which had provoked the anaphylactic reaction upon the ingestion, could be completely abolished after inhibition with purified cod parvalbumin.

The clinical sensitivity of fish allergic patients to frog parvalbumin was further assessed by skin prick tests using recombinant parvalbumin α and β from *R. esculenta* on three fish allergic patients. All of the patients tested reacted to recombinant *R. esculenta* parvalbumin β at a concentration of 0.7 $\mu\text{g/ml}$.

The IgE cross-reactions described in this paper were directed from fish to frog and mainly from fish parvalbumin to frog parvalbumin β . A frog allergic patient whose case was recently published (14) reacted very specifically to the parvalbumin α molecule of the frog species from Indonesia to which he was allergic. He did not show any cross-reactivity to other frogs as for instance *R. esculenta*, nor to fish as for instance cod or salmon. Parvalbumins have been shown to be major allergens in fish. They have also been shown to be a primary allergen in frog. Now they have been shown to be cross-reacting allergens between fish and frog. As parvalbumins are present in the fast twitch muscles of many vertebrates it might be rewarding to check whether, hitherto unexplained allergic reactions to meat are not due to a sensitization to parvalbumins giving parvalbumins the status of a new family of cross-reactive allergens.

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